

# EXHIBIT E

# Defective Bone Formation and Anabolic Response to Exogenous Estrogen in Mice with Targeted Disruption of Endothelial Nitric Oxide Synthase\*

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## ABSTRACT

Nitric oxide (NO) is a pleiotropic signaling molecule that is produced by bone cells constitutively and in response to diverse stimuli such as proinflammatory cytokines, mechanical strain, and sex hormones. Endothelial nitric oxide synthase (eNOS) is the predominant NOS isoform expressed in bone, but its physiological role in regulating bone metabolism remains unclear. Here we studied various aspects of bone metabolism in female mice with targeted disruption of the eNOS gene. Mice with eNOS deficiency (eNOS KO) had reduced bone mineral density, and cortical thinning when compared with WT controls and histomorphometric analysis of bone revealed profound ab-

normalities of bone formation, with reduced osteoblast numbers, surfaces and mineral apposition rate. Studies *in vitro* showed that osteoblasts derived from eNOS KO mice had reduced rates of growth when compared with WT and were less well differentiated as reflected by lower levels of alkaline phosphatase activity. Mice with eNOS deficiency lost bone normally following ovariectomy but exhibited a significantly blunted anabolic response to high dose exogenous estrogen. We conclude that the eNOS pathway plays an essential role in regulating bone mass and bone turnover by modulating osteoblast function. (*Endocrinology* 142: 760–766, 2001)

**N**ITRIC OXIDE (NO) is a pleiotropic signaling molecule that has potent effects on osteoblast and osteoclast activity *in vitro* (1). Bone cells produce NO in response to a variety of stimuli including proinflammatory cytokines (2–6), mechanical loading (7), fluid flow (8, 9), and estrogen (10). Studies *in vitro* have indicated that NO has biphasic effects on both osteoclast (5, 11, 12) and osteoblast activity (2, 6, 13), whereas studies *in vivo* have suggested a possible role for constitutive NO production in regulating bone mass and bone turnover (14–19). Endothelial nitric oxide synthase (eNOS) is the isoform most widely expressed in bone, but its role in regulating bone metabolism remains unclear because the inhibitors that have generally been used to probe NOS function have inhibitory effects on all NOS isoforms and inhibitory effects on other L-arginine-dependent metabolic pathways distinct from NOS (20). In view of this, we attempted to clarify the role of the eNOS pathway in regulating bone turnover, by investigating various aspects of bone metabolism and bone cell function in mice with targeted disruption of the eNOS gene.

## Materials and Methods

### Animals

Mice with targeted inactivation of eNOS were generated as previously described by Gödecke and colleagues (21) by deleting the se-

quences coding for the NADPH binding site in exons 24 and 25 with a neomycin-resistant cassette. E14–1 embryonic stem cells targeted with the eNOS gene construct were microinjected into blastocysts of C57BL/6 mice to generate chimeric animals. Male chimeric animals were bred with female C57BL/6 mice for 7 generations to establish homozygous inbred lines of eNOS deficient mice (eNOS KO) and wild-type (WT) C57BL/6 controls. All experiments were performed in accordance with UK Home Office guidelines on 8-week-old adult female mice unless stated otherwise.

### Bone mineral density measurements

Measurements of volumetric bone mineral density (BMD) were performed by peripheral quantitative computed tomography (pQCT) with an XCT Research M pQCT bone densitometer (Stratec Medizintechnik, Pforzheim, Germany) using a voxel size of 100  $\mu$ m and software version 5.14. Quality assurance measurements were performed daily with a plexiglass coated (PVC) phantom according to the manufacturer's instructions. *Ex vivo* measurements were carried out on the left tibial bones and calvarial bones of mice that had been dissected free of soft tissues. *In vivo* measurements were performed at the tibial metaphysis of mice which had been anesthetized with 0.2% Rompun (Bayer plc, Bury St Edmunds, UK) and 10 mg/ml Vetalar V (Pharmacia & Upjohn, Inc., Crawley, UK), with the animals placed in a prostrate position on the scanning platform. All scans were performed at the proximal tibial metaphysis 0.9 mm distal to the growth plate. The precision of *ex vivo* BMD measurements was evaluated by performing 10 repeat measurements of the same bone after repositioning and was found to be 1.19% for total BMD, 3.53% for trabecular BMD, and 1.04% for cortical BMD. Corresponding figures for the *in vivo* measurements at the tibiae were 2.59%, 5.28%, and 1.76%. Measurements of bone length were made using Vernier calipers (Vaduz, Holland, PAV 0–25).

### Bone histomorphometry

Histomorphometric measurements were performed on sections from the metaphysis of the left femur distal to the epiphyseal growth plate using a Leica Corp. Q500 MC image analysis system (Leica Corp., Cam-

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bridge, UK). Bones were fixed in 4% buffered formalin/saline (pH 7.4) and embedded in methyl methacrylate. 4  $\mu$ m longitudinal sections were prepared, stained with Von Kossa and Paragon and read in the metaphysis, distal to the epiphyseal growth plate at 20 $\times$  magnification. Histomorphometric variables were expressed as previously described (22). The mineral apposition rate (MAR) of bone was assessed on unstained decalcified cross-sections using fluorescent microscopy in femur obtained from 11-week-old mice that had received ip injections of 40 mg/kg calcein green (Sigma, Poole, UK) 10 and 4 days before they were killed.

#### Cell culture

Primary osteoblast cultures were established from neonatal eNOS KO and WT mice by sequential collagenase/EDTA digestion of calvarial bones, and cells cultured in 75-cm<sup>2</sup> flasks in  $\alpha$ -MEM with 10% FCS until confluent. The cells were trypsinized and seeded into 96-well tissue culture plates at a density of 10<sup>4</sup>/well in phenol red-free culture medium with charcoal stripped FCS and allowed to adhere overnight. Fresh medium containing test substances was added and the cultures were continued for 48 h. Cell growth was assessed by the 3-(4,5-dimethylthiazol-2-yl) (MTT) assay (Sigma) (23) and alkaline phosphatase activity in the cell layer was determined by a colorimetric assay using p-nitrophenol phosphate as a substrate (Sigma), as previously described (24), and corrected for cell number.

#### Ovariectomy and estrogen treatment

Bilateral ovariectomy (Ovx) was performed under general anesthesia. Sham ovariectomy (Sham) was similarly performed but with externalization and replacement of the ovaries. Estrogen treatment was delivered by sc implantation of 1  $\mu$ g or 10  $\mu$ g 17 $\beta$ -Estradiol (E<sub>2</sub>) slow release pellets which deliver 0.047  $\mu$ g and 0.476  $\mu$ g estradiol per day for 21 days, respectively. Placebo pellets containing vehicle only were used as controls (Innovative Research of America, Sarasota, FL). These doses of estrogen are similar to those used by previous workers who have investigated the effects of exogenous estrogen on BMD in ovariectomized mice (25–27). BMD measurements were carried out immediately after OvX or sham and these were repeated 21 days later on termination of the experiment. To correct for the effects of skeletal growth, changes in BMD and other variables during the 21-day study period were subtracted from the changes observed in sham operated animals of the same genotype.

#### Plasma 17 $\beta$ -estradiol measurements

Measurements of 17 $\beta$ -estradiol were made on blood samples obtained at cardiac puncture by RIA using a sheep polyclonal antibody (DPC Ltd., Llanberis, Wales, UK).

#### Statistical analyses

Statistical analyses were performed using SPSS, Inc. version 9.0. Between group differences were assessed by Student's *t* test for pairwise comparisons and ANOVA with Tukey's or Dunnett's post-test for mul-

tiples comparisons. Results of the cell culture experiments were analyzed by a General Linear Model (GLM) ANOVA using treatment and genotype as grouping variables. All data are presented as means  $\pm$  SEM unless otherwise stated. Two-sided *P* values of less than 0.05 were considered significant.

### Results

#### Bone mineral density and bone geometry

Studies of the tibial metaphysis by pQCT showed that 8-week-old eNOS KO mice had a 13.3% lower total BMD than age matched WT controls, due to a reduction in both cortical (6.5%) and trabecular components (18.3%) (Table 1). These abnormalities were accompanied by a significant reduction in cortical thickness, a significant increase in endosteal circumference, and a non-significant increase in periosteal circumference. The strength-strain index, which is a predicted biomechanical variable that reflects the bending strength of bone (28), was also significantly reduced in eNOS KO mice when compared with WT controls, consistent with the abnormalities of cortical width and endosteal circumference. The polar cross-sectional moment of inertia (pCSMI), which reflects the torsional strength of bone (28), was also lower in eNOS KO mice when compared with WT, but this difference was not significant. Similar abnormalities were observed on analysis of bones from 20-week-old animals (Table 1). Body weight in age matched eNOS KO and WT mice was similar (21.84  $\pm$  0.48 g vs. 22.61  $\pm$  0.63 g; *P* = 0.34). Calvarial bones of 19-day-old eNOS KO mice also showed reduced total BMD values when compared with WT controls (308.7  $\pm$  7.3 mg/cm<sup>3</sup> vs. 357.2  $\pm$  3.7 mg/cm<sup>3</sup> (*P* < 0.001).

#### Bone histomorphometry

Histological examination of bone supported the pQCT results in showing evidence of osteopenia in eNOS KO mice when compared with WT controls (Fig. 1). Quantitative histomorphometry at the distal femoral metaphysis (Table 2) showed that eNOS KO mice had significantly reduced trabecular bone volume and cortical thickness when compared with WT as well as significantly reduced osteoblast numbers, osteoblast surfaces, and mineral apposition rate. There was no significant difference in osteoclast numbers or resorption surfaces between eNOS KO and WT mice, however (Table 2), suggesting that the reduction of bone mass was primarily

**TABLE 1.** Bone mineral density, skeletal geometry, and predicted biochemical properties of bone in eNOS KO and WT mice

pQCT Parameters (units)	WT		eNOS KO	
	8 weeks		20 weeks	
Total bone density (mg/cm <sup>3</sup> )	438.6 $\pm$ 9.5		380.4 $\pm$ 8.3 <sup>b</sup>	536.4 $\pm$ 11.8
Trabecular bone density (mg/cm <sup>3</sup> )	281.3 $\pm$ 10.7		229.8 $\pm$ 14.1 <sup>a</sup>	209.7 $\pm$ 16.8
Cortical bone density (mg/cm <sup>3</sup> )	738.4 $\pm$ 11.7		690.3 $\pm$ 4.6 <sup>b</sup>	901.5 $\pm$ 7.7
Cortical thickness (mm)	0.187 $\pm$ 0.006		0.134 $\pm$ 0.013 <sup>b</sup>	0.172 $\pm$ 0.008
Endosteal circumference (mm)	5.316 $\pm$ 0.150		5.890 $\pm$ 0.149 <sup>a</sup>	5.154 $\pm$ 0.096
Periosteal circumference (mm)	6.491 $\pm$ 0.124		6.730 $\pm$ 0.132	6.235 $\pm$ 0.113
Polar cross-sectional moment of inertia (mm <sup>4</sup> )	0.740 $\pm$ 0.029		0.644 $\pm$ 0.051	0.670 $\pm$ 0.049
Strength strain index (mm <sup>3</sup> )	0.657 $\pm$ 0.020		0.510 $\pm$ 0.042 <sup>b</sup>	0.566 $\pm$ 0.042
				0.413 $\pm$ 0.047 <sup>a</sup>

*Ex vivo* measurements of volumetric BMD were carried out using peripheral quantitative computed tomography on left tibiae that had been dissected free of soft tissues as described in the *Materials and Methods* section. Values represent means  $\pm$  SEM of 7 mice per group. Significant differences between eNOS KO mice compared with age-matched WT controls are represented by <sup>a</sup> *P* < 0.05 and <sup>b</sup> *P* < 0.01.

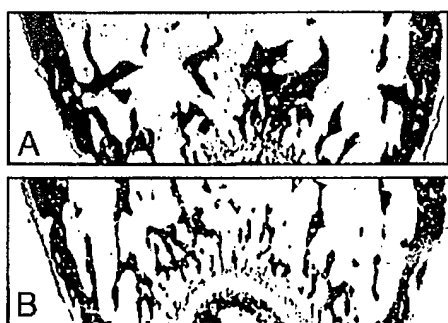


FIG. 1. Bone phenotype in eNOS KO mice. Four-micrometer longitudinal bone sections from the distal femur of eNOS KO and WT mice stained with Von Kossa and Paragon. A, WT. B, eNOS KO. Lower trabecular bone density and cortical bone thickness are observed in the eNOS KO mouse.

TABLE 2. Bone histomorphometric parameters of eNOS KO and WT femur

Bone Histomorphometric Parameters (units)	eNOS WT	eNOS KO	P value
BV/TV (%)	30.81 ± 4.13	18.19 ± 2.51	0.023
Trab.Th (mm)	0.017 ± 0.002	0.015 ± 0.001	0.398
Ct.Th (mm)	0.162 ± 0.015	0.121 ± 0.009	0.047
N.Ob.S (/mm)	3.85 ± 0.35	2.38 ± 0.47	0.027
N.Ob (/mm <sup>2</sup> )	86.1 ± 30.1	38.5 ± 6.0	0.000
Ob.S/BS (%)	4.72 ± 0.79	2.50 ± 0.47	0.033
Osteoid width (μm)	5.37 ± 0.29	5.25 ± 0.41	0.818
MAR (μm/day)	2.67 ± 0.30	2.02 ± 0.18	0.024
N.Oc.S (/mm)	0.73 ± 0.09	0.91 ± 0.11	0.220
N.Oc (/mm <sup>2</sup> )	16.0 ± 2.1	15.1 ± 2.0	0.746
Oc.S (%)	3.65 ± 0.83	4.21 ± 0.74	0.623
ES (%)	4.73 ± 0.94	5.53 ± 0.84	0.540

Bone histomorphometry was performed on 4 μm longitudinal bone sections from age-matched eNOS KO and WT mice stained with Von Kossa and Paragon in 4–5 fields as described in the Methods section. The mineral apposition rate was measured in unstained decalcified cross-sections of femur. Abbreviations: trabecular bone volume/total tissue volume (BV/TV), trabecular bone thickness (Trab.Th), cortical bone thickness (Ct.Th), osteoblast number per bone surface unit (N.Ob.S), osteoblast number (N.Ob) and osteoblast surface (Ob.S/BS), mineral apposition rate (MAR), osteoclast number per bone surface unit (N.Oc.S), osteoclast number (N.Oc), active resorption surface (Oc.S) and total resorption surface (ES). Values represent means ± SEM of seven bones per group.

due to a defect in osteoblast activity and bone formation, rather than an increase in bone resorption.

#### Cell culture

Alkaline phosphatase (AP) activity was significantly lower under all culture conditions ( $P < 0.0001$ ) in eNOS KO cultures when compared with WT (Fig. 2A). Although PTH caused a 2.2-fold stimulation of AP activity in WT osteoblasts, there was no significant response in eNOS KO osteoblasts. 17β-estradiol, 1,25 dihydroxyvitamin D3 and PTH (Sigma) had no significant effect on cell growth in either eNOS KO or WT cultures, but the growth of osteoblasts derived from eNOS KO animals was significantly depressed ( $P < 0.0001$ ) when compared with WT under all culture conditions (Fig. 2B).

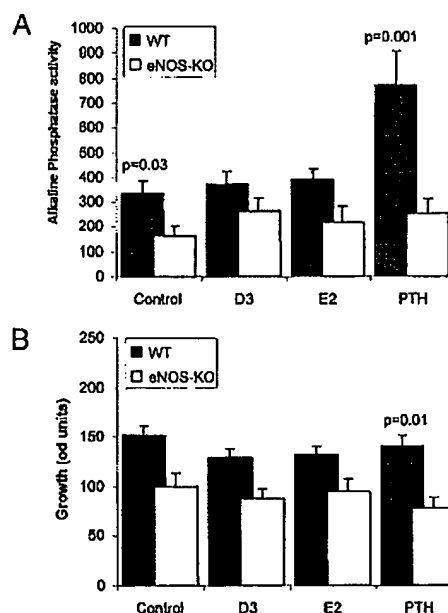


FIG. 2. Osteoblast function in eNOS KO and WT mice. A, Alkaline phosphatase activity (means ± SEM) corrected for cell number in eNOS KO and WT osteoblasts. Significant pairwise differences for individual stimuli are indicated. The difference between genotypes was highly significant overall as assessed by ANOVA-GLM ( $P < 0.0001$ ). B, Cell growth (means ± SEM), assessed by MTT assay in eNOS KO and WT osteoblasts. Significant pairwise differences for individual stimuli are indicated. The difference between genotypes was highly significant overall as assessed by ANOVA-GLM analysis ( $P = 0.019$ ). The data shown are from three separate experiments with six replicate wells per experiment. E2, 17β-estradiol  $10^{-8}$  M; D3, 1,25 dihydroxyvitamin D3  $10^{-8}$  M; PTH, 1–34 fragment of human recombinant PTH  $4 \times 10^{-8}$  M.

#### Response to ovariectomy and exogenous estrogen

There was no significant difference between basal circulating concentrations of 17β-estradiol in eNOS KO and WT mice ( $103.6 \pm 19.0$  pm vs.  $101.9 \pm 9.5$  pm;  $P = 0.93$ ). As expected, Ovx reduced BMD, BMC, and uterine weight and increased body weight when compared with sham, but there was no significant difference in the response of these variables between eNOS KO and WT mice (Table 3 and Fig. 3). Low dose estrogen (1 μg/21 days) partially prevented Ovx induced bone loss in both eNOS KO and WT mice but did not restore values to those observed in sham controls. High dose estrogen (10 μg/21 days) markedly increased BMD, BMC, cortical thickness, periosteal circumference, and pCSMI in both genotype groups, but the magnitude of the response was significantly blunted in eNOS KO animals when compared with WT (Figs. 3 and 4). Although eNOS KO mice had a reduced percentage gain in body weight with high dose estrogen, this was not significantly different from the response in WT. Moreover, the differences in anabolic response to high dose estrogen remained significant for trabecular BMD, total, trabecular and cortical BMC, cortical thickness, periosteal circumference, endosteal circumference, and pCSMI after correcting for changes in body weight by GLM ANOVA (data not shown).

TABLE 3. Response of body weight and uterine weight to ovariectomy and estrogen treatment in eNOS KO and WT mice

Treatment	Genotype	Sham	Ovx	Ovx + E <sub>2</sub> (1 µg)	Ovx + E <sub>2</sub> (10 µg)
Gain in body weight (%)	eNOS WT	9.1 ± 0.8	18.0 ± 0.9 <sup>a</sup>	17.8 ± 3.2	12.8 ± 1.3
	eNOS KO	8.0 ± 2.0	18.3 ± 2.0 <sup>a</sup>	17.9 ± 2.2	6.6 ± 2.0 <sup>b</sup>
Uterus weight (mg)	eNOS WT	75.8 ± 7.3	16.9 ± 2.5 <sup>a</sup>	15.6 ± 4.9	136.9 ± 25.2 <sup>b</sup>
	eNOS KO	87.4 ± 7.0	22.9 ± 1.8 <sup>a</sup>	36.4 ± 10.8	161.0 ± 20.1 <sup>b</sup>

Values represent means ± SEM of 7–25 mice per group. Significant values are shown as <sup>a</sup>  $P < 0.01$  vs. Sham of same genotype, <sup>b</sup>  $P < 0.01$  vs. Ovx of same genotype.

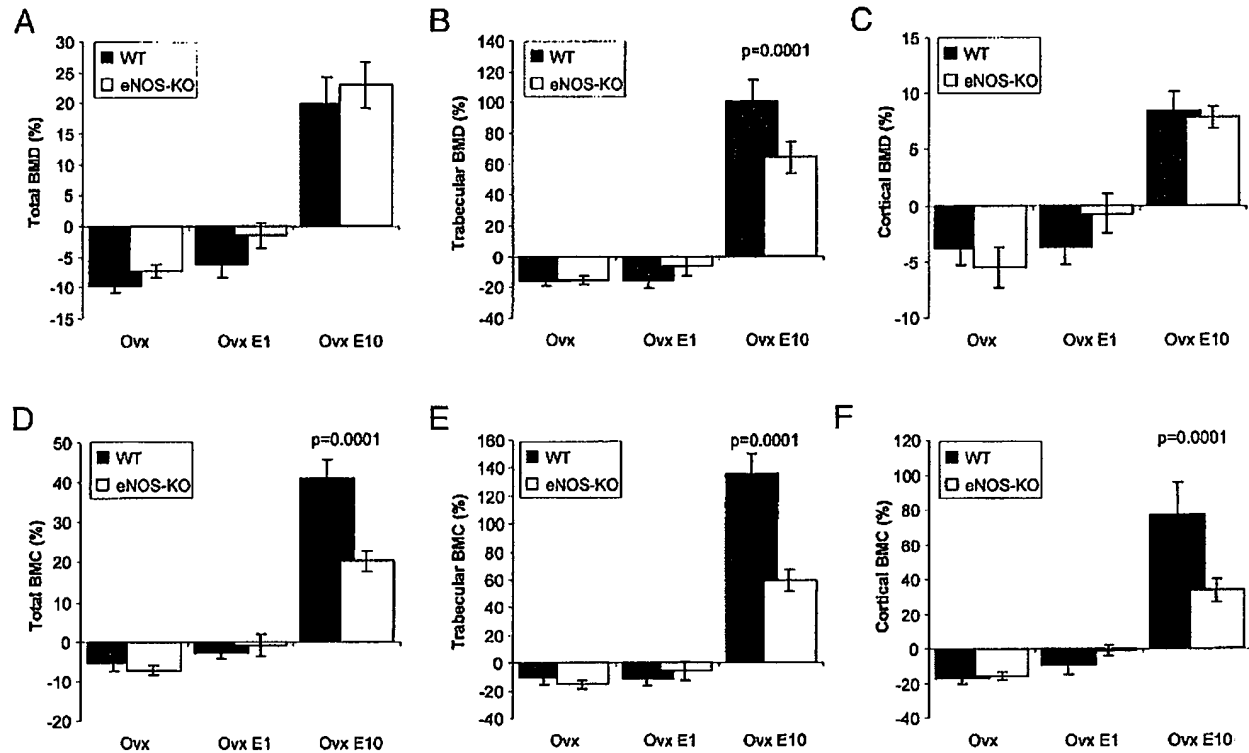


FIG. 3. Response of BMD and BMC to ovariectomy and estrogen replacement in eNOS and WT mice. Volumetric BMD and BMC were measured at the tibial metaphysis by pQCT in eNOS KO and WT mice undergoing ovariectomy (Ovx) and treatment with 1 µg or 10 µg estrogen pellets (Ovx E1 and Ovx E10). A–C, The response of BMD. D–F, The response of BMC. Values are means ± SEM of 7–25 animals per group and are expressed as a percentage change in relation to the values observed in WT sham operated controls of the same genotype to correct for the effects of skeletal growth. Pairwise differences between eNOS KO and WT mice are indicated by the  $P$  values. There were significant differences for all BMC and BMD measurements between sham and Ovx ( $P < 0.01$ ); sham and Ovx E10 ( $P < 0.001$ ) and between Ovx and Ovx E10 ( $P < 0.001$ ) within each genotype. Ovx E1 did not differ from Ovx for any measurement in either genotype group.

### Discussion

Constitutive production of NO derived from the eNOS pathway has been suggested to play a role in regulating bone cell function and bone turnover. Several groups have shown that eNOS is the predominant isoform expressed in adult bone and bone-derived cells (29, 30), and studies *in vitro* (10) and *in vivo* (14) have indicated that eNOS may act as a mediator of estrogen actions in bone as well as the cardiovascular system (31). The physiological role of the eNOS pathway in bone has hitherto been difficult to assess, however, because the NOS inhibitors that have been used to probe NO function have inhibitory effects on all NOS isoforms and on L-arginine-dependent pathways other than the NOS pathway (20). This has resulted in marked discrepancies between *in vitro* studies such that constitutive NO production has been variously suggested to be

essential (13) or nonessential (32) for osteoblast function and to be essential (33) or inhibitory (16, 34) for osteoclast function depending on the model system and NOS inhibitor used. Studies of NOS inhibitors *in vivo* have also yielded conflicting results. For example, it has been suggested that constitutive production of NO may inhibit osteoclast activity and protect against ovariectomy induced bone loss on the basis that aminoguanidine caused accelerated bone loss in rats (16, 17). Other studies have shown that different NOS inhibitors such as L-NMMA and L-NAME have little or no effect on bone density, ovariectomy induced bone loss or osteoclast activity, however (14, 15, 17, 35). The use of mice with targeted inactivation of eNOS circumvents these problems and allows us to more clearly define the role that the eNOS pathway plays in regulating bone mass and bone turnover.

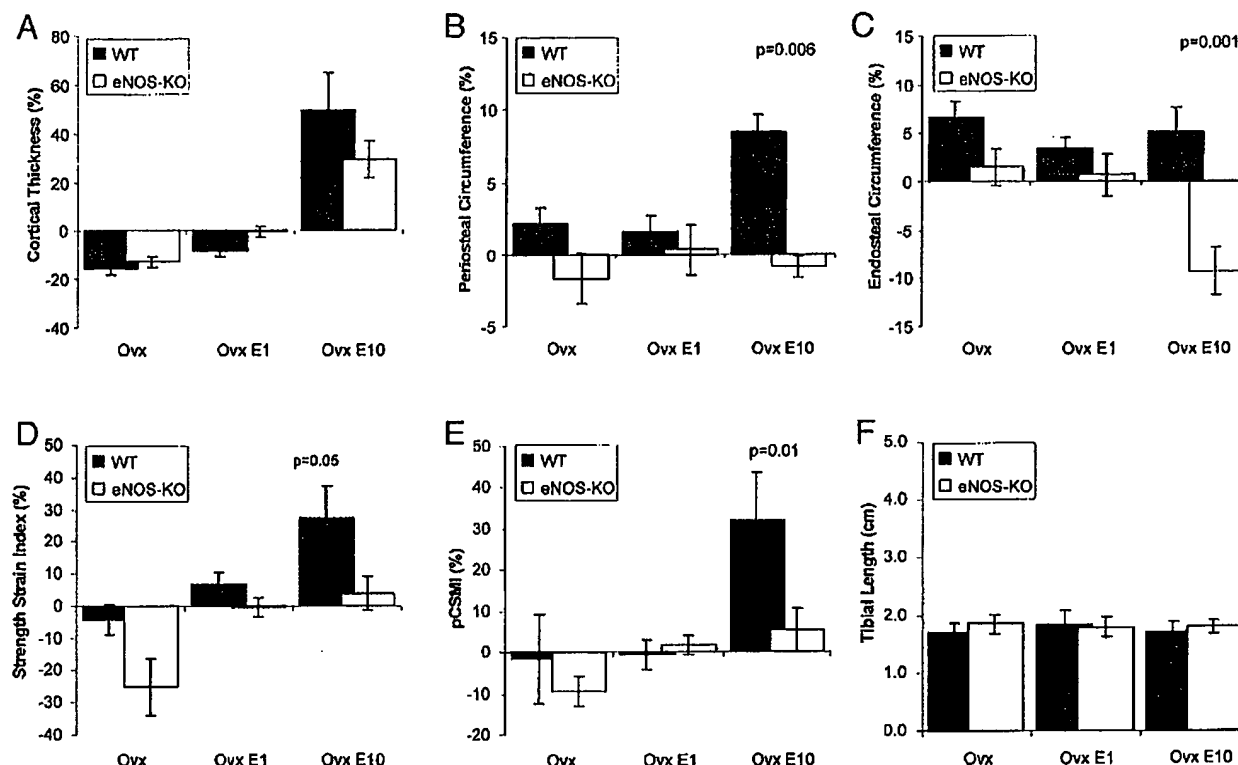


FIG. 4. Response of bone geometry and biomechanical variables to Ovx and estrogen replacement. Geometric and predicted biomechanical variables were measured at the tibial metaphysis by pQCT in eNOS KO and WT mice undergoing ovariectomy (Ovx) and treatment with 1  $\mu$ g or 10  $\mu$ g estrogen pellets (Ovx E1 and Ovx E10). A–C, The response of cortical thickness, periosteal circumference and endosteal circumference; D–F, the response of strength strain index, polar cross-sectional moment of inertia (pCSMI) and tibial length. Values are means  $\pm$  SEM of 7–25 animals per group and are expressed as a percentage change in relation to the values observed in WT sham operated animals, to correct for the effects of skeletal growth, with the exception of tibial length, which was measured in cm on termination of the experiment. Pairwise differences between eNOS KO and WT groups are indicated by the *P* values. There were significant differences within each genotype between sham and Ovx (*P* < 0.01); sham and Ovx E10 (*P* < 0.001) and between Ovx and Ovx E10 (*P* < 0.001) for cortical thickness and endosteal circumference. Ovx E1 did not differ from Ovx for any variable in either genotype group.

Studies with peripheral quantitative computed tomography showed that eNOS KO mice had significantly reduced bone mineral density in both cortical and trabecular compartments when compared with WT controls as well as a reduction in cortical thickness, and an increase in endosteal circumference. These abnormalities in bone density and bone geometry were accompanied by a significant reduction in strength strain index, which is a predicted variable reflecting the bending strength of bone (28). The abnormalities of bone mass and bone structure noted in eNOS KO mice were not restricted to a single skeletal site or to a specific stage in life, because we observed a reduction in density of the calvarial bones by pQCT at 19 days of age and found that the abnormalities of BMD in the tibiae persisted in mice of up to 20 weeks of age. These observations indicate that eNOS deficiency has generalized effects on the skeleton that are apparent early in life and persist to the attainment of peak bone mass and beyond.

Bone histomorphometric studies were undertaken to define the mechanisms responsible for the reduction in bone mass and these showed a profound defect in bone formation in eNOS KO mice when compared with WT, as reflected by

reductions in osteoblast number, osteoblast surface and mineral apposition rate. Although osteoclast numbers were marginally higher in eNOS KO mice when compared with WT controls, there was no significant difference between the genotypes in this parameter or any of the other bone resorption indices studied. These data indicate that the reduced bone mass observed in eNOS KO mice is primarily due to a defect in bone formation rather than an increase in bone resorption. Although we cannot completely exclude the possibility that subtle differences in osteoclast activity may have contributed to the phenotype observed, our data strongly suggest that the marked increases in osteoclast activity noted in rodents that have been given the NOS inhibitor aminoguanidine (16, 17) is due to inhibition of other NOS isoforms or other L-arginine-dependent metabolic pathways rather than eNOS inhibition.

Consistent with the histomorphometric studies, cultured osteoblasts from eNOS KO mice had significantly reduced rates of growth and alkaline phosphatase activity when compared with WT osteoblasts. Although further studies will be required to investigate the nature of the osteoblast defect in more detail, these data clearly show that the eNOS pathway

plays an essential role in regulating osteoblast differentiation and function *in vitro* and *in vivo*. Because eNOS deficiency results in hypertension, it is conceivable that alterations in the vascular supply to bone during growth and development may also have contributed to the phenotype observed. This possibility is difficult to address directly in the absence of validated methods for assessing bone vasculature or blood supply during development in mice. Evidence from other sources indicates that hypertension alone is unlikely to explain the phenotype observed however. Firstly, reduced osteoblast activity has not been observed in other rodents with genetically determined hypertension such as the spontaneously hypertensive rat, which instead shows evidence of secondary hyperparathyroidism and increased bone turnover (36, 37). This observation argues against a direct inhibitory effect of raised blood pressure on osteoblast function and bone formation. Secondly, one cannot invoke reduced blood flow as a cause for the persistent abnormalities of osteoblast function which were noted *in vitro*. From this it would appear likely that the primary reason for defective osteoblast function in eNOS KO animals is deficiency of eNOS, rather than the effects of hypertension on bone during bone development.

One of the most striking abnormalities that we observed in the present study was marked blunting of the anabolic response to high dose estrogen in ovariectomized eNOS-deficient mice when compared with WT controls. Previous studies have indicated that this response is primarily due to increased bone formation (26), which is consistent with the histomorphometric and cell culture data showing that eNOS deficiency is associated with impaired osteoblast activity and defective bone formation. The abnormal response of alkaline phosphatase to PTH treatment *in vitro* suggests that the defect in osteoblast function may be a generalized one rather than estrogen specific, but further studies will be required to determine whether the response to other anabolic agents *in vivo* is similarly impaired in eNOS KO mice.

We observed no difference in the degree of bone loss or response of geometric parameters to ovariectomy in eNOS KO mice when compared with WT controls, which is consistent with a role for NO-independent pathways in this response (38, 39), nor did we observe differences in the response to low dose estrogen. Although our studies were carried out in female mice, because of the previously demonstrated link between eNOS and estrogen responsiveness in bone (10), similar studies by other workers have shown evidence of osteopenia in male mice with eNOS deficiency indicating that the effects on bone are not gender specific (40).

It is recognized that BMD values can differ markedly between different mouse strains as the result of genetic influences (41). Differences in genetic background cannot readily be invoked as an explanation for the effects observed here because these differences were minimized by breeding F1 heterozygotes for 7 generations onto the background C57BL/6 strain to obtain an inbred colony before the experiments commenced. In support of this view, recent work also showed evidence of reduced trabecular bone volume and impaired osteoblast function in a different colony of mice with eNOS deficiency (40).

In conclusion, our results show that eNOS is essential for

normal osteoblast differentiation and function and that eNOS deficiency is associated with reduced bone mass and an impaired anabolic response to high dose estrogen. These data suggest that eNOS plays a key role in regulating osteoblast function and raises the possibility that the eNOS pathway might represent a novel target for pharmacological modulation to increase bone formation.

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